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14. ABSTRACT
The purpose of this project was to determine whether mitotic spindle position differs in benign versus malignant breast tissues, and to test whether estradiol alters spindle position. The most significant findings are that mammary tissue shows planar spindle orientation. Information about spindle position in breast cancer tissues for comparison is still pending, awaiting an additional collaboration. The other significant finding is that spindle orientation appeared random in MCF10-A cells and an endometrial cancer cell line (Ishikawa), but regulated in three dimensional cysts of MDCK cells. This regulation is sensitive to disruption of actin and microtubules, and to EGTA treatment. Now that the postdoc working on the project has left, I want to re-visit the experiments with MCF-10A cells using serum free media.

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Introduction

Breast cancer is a devastating disease and a major public health problem. Estrogen Receptor (ER) signaling stimulates breast cancer development, and anti-estrogen therapies are a mainstay of therapy. In the uterus, Estradiol treatment was shown to cause rotation of mitotic spindles relative to the tissue plane, an established feature of planar polarity and a major determinant of epithelial tissue morphology (*1*). This proposal sought to determine the connection between ER signaling and microtubule regulation in the breast. We tested hypotheses that breast tumors show abnormal mitotic spindle positioning, and that ER signaling alters spindle position in cultured breast epithelial cells. A finding of estrogen-induced changes in spindle orientation could increase our understanding of mechanisms of ductal hypertrophy and breast cancer development.

Body

Aim 1/Task 1 was to determine whether mitotic spindle position differs between benign and malignant breast tissues. As described above, orientation of the spindle has the potential to affect breast cancer development. This Aim looked at spindle orientation in vivo, to determine if it is altered in tumors. It requires development of techniques to image spindles in breast tissues and to analyze the three-dimensional images. We acquired breast samples from our Pathology Department but these were not mitotically active enough for analysis. We used mouse tissues from another investigator, in which the phase of the hormonal cycle was known.

1a) Begin accumulating samples from Pathology Dept. for analysis

Benign breast tissues from seven reduction mammoplasties were obtained from the Pathology Department for analysis. We were able to obtain a small number of tumors (de-identified) from our Pathology Department. These were insufficient for analysis because the tissues were not mitotically active enough for visualization of enough spindles for quantitative analysis. In the meantime, we used human intestinal tissue, which we know to be mitotically active, and optimized methods for visualizing mitotic spindles and calculating spindle orientation angle.

1b) Pilot experiments for estrogen receptor (ER) staining and microtubule staining

In adult virgin mice in proestrus, we found the breast ductal tissues to be sufficiently proliferative to allow for mitotic spindle imaging. We focused on spindles alongside ducts, rather than at acini, because it was more straightforward to assign the duct vector and unambiguously determine spindle angle relative to the duct wall (Figure 1).

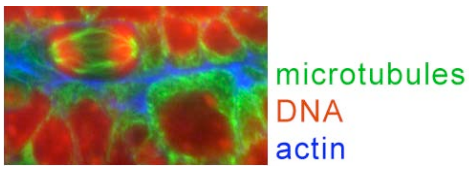

Figure 1. Staining of adult virgin mouse mammary tissue in proestrus shows mitotic spindles are parallel to the duct lumen. Tissues were excised, fixed, and immunofluorescence for microtubules (green) DNA (red) and phalloidin staining for actin (blue) performed to show the spindles, chromatin, and apical surfaces of cells along the duct lumen. This image shows that the spindle is clearly aligned parallel to the duct lumen (low angle). Quantitation is shown in the following section.

1c) Staining of breast samples for microtubules and ER

As we were able to image spindles in mouse mammary tissues, we analyzed their angles, to determine whether spindle orientation was regulated. We found that spindle angles in these tissues were highly regulated, with a mean angle of 16° (n = 56 spindles from four animals), as shown in Figure 2. This result confirms that spindle angle is highly regulated in vivo. Spindle orientation parallel to the duct lumen would lead to duct lengthening, or, when balanced by apoptosis, to homeostasis of duct morphology, rather than to duct hypertrophy or branching.

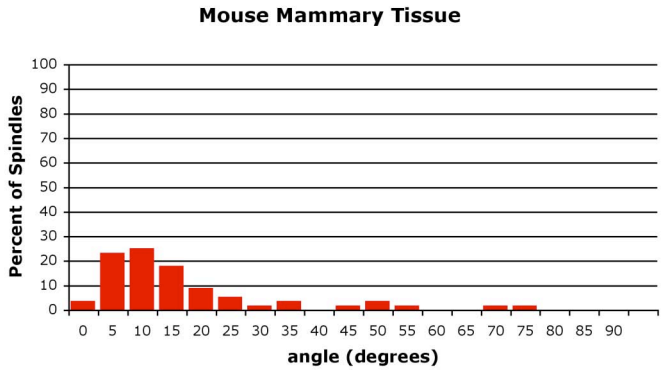

Figure 2. Spindle angle analysis from adult virgin mouse mammary tissue shows regulated spindle orientation. Confocal image stacks from acini were analyzed and spindle angle calculated. Spindles were oriented parallel to the duct lumen, with a mean angle of 16° (n = 56 spindles from four animals). This demonstrates regulated spindle orientation in mammary tissue in vivo.

1d) Analysis of spindle angles from immunofluorescence images

We hope next to measure spindle angles in a mouse mammary tumor model, to determine if they are abnormally oriented. It took a while but we have finally gotten a Material Transfer Agreement to analyze adenocarcinomas from MMTV-tTA/tet-op-ERalpha/tet-op-SimianVirus 40 T antigen transgenic mice from Dr. Shiffert's lab. This will commence when the tissues arrive here.

In the meantime we will continue to pursue appropriate and usable human tissues, as described above.

Aim 2/Task 2 is to determine whether Estradiol exposure affects spindle positioning in cultured MCF-10A acini. Regardless of findings in Aim 1, we propose to test the effects of Estradiol on spindle orientation in cultured cells. We believe it is important to use non-malignant cells lines when possible, to avoid potentially confounding effects of mutations in the tumor cell lines on spindle orientation. MCF-10A cells are useful for this purpose, but they lack ER expression. Recently, another group has engineered MCF-10A cells to express ERα and to undergo physiological ER signaling and provided the cells to us (*2*). The other important variable to establish in cultured cells is epithelial polarity.

2a) Grow control acini from MCF10-A cells.

We grew MCF-10A acini on Matrigel beds as described in (*3*). We found that Matrigel did not spread properly in standard 8 well slide plates, so we devised a home-made chamber that allowed us to plate multiple sets on acini on one microscope slide. We tested the growth response of MCF-10A cells expressing the ER (called ERIN9 cells) and confirmed physiologic ER signaling in these cells (data not shown). These were also able to form acini when grown on a Matrigel bed.

2b) Immunofluorescence on control acini for microtubules.

We preformed immunofluorescence for microtubules and γ-tubulin, as well as DNA staining, to image spindles in the acini. Figure 3 shows an example of a representative acinus. This spindle was mis-oriented relative to the acinar lumen.

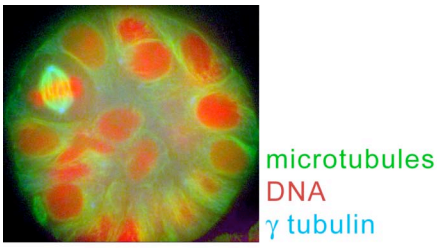

Figure 3. Example of MCF-10A acinus demonstrating mis-oriented spindle. MCF-10A cells were grown on Matrigel beds, and acini were allowed to form. Acini were fixed and immunofluorescence for tubulin (green) and γ tubulin (blue) and staining for DNA (red) performed for visualization of spindle, spindle poles, and chromosomes, respectively. Three-dimensional confocal stacks were used to measure the X, Y, Z coordinates of the spindle poles and to calculate the spindle angle relative to the apical cell surface in the center (lumen) of the acinus, which is not visible in this confocal slice. The spindle shown here is mis-oriented.

To determine whether spindle orientation was regulated in the MCF-10A and ERIN9 acini, we imaged multiple spindles and calculated their angle relative to the acinar lumen. We were surprised to find that spindles were randomly oriented under these experimental conditions, indicating that the orientation process was not regulated (Figure 4).

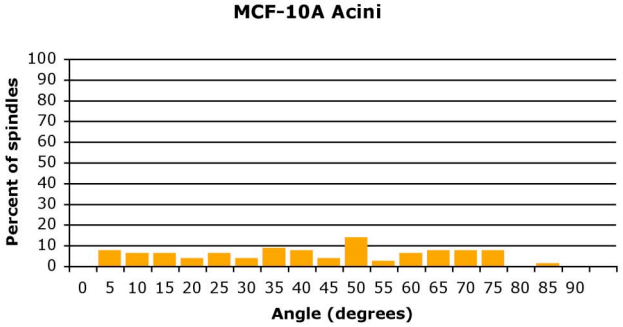

Figure 4. Spindle angle analysis from MCF-10A acini shows random spindle orientation. Confocal image stacks from acini were analyzed and spindle angle calculated (n = 80 spindles). Spindles were randomly oriented across a range of angles, indicating that the process was not regulated under these experimental conditions.

We considered several explanations for this lack of spindle angle regulation, including the known lack of tight junctions in MCF-10A (and therefore ERIN9) cells, the composition of the Matrigel, and the fixation conditions (*4*). We believe the most likely explanation is that at the time-point that acini became truly polarized, they were no longer proliferative. We therefore were likely collecting spindles in acini that were not truly polarized. Because spindle orientation is in part controlled by cell polarization, we suspect that this created a situation in which spindles were not orientated because cell polarization was incomplete. In previous projects undertaken by the Principal Investigator, this was not a technical problem, but in this project it appeared to be a problem without a clear explanation. Our solution was to explore alternative cell lines, and to explore alternative ways of culturing MCF-10A cells.

In seeking an alternative system in which to study spindle control by Estradiol, we examined several established cell lines. We found that spindles were highly regulated in the kidney cell line MDCKII, as has been published, but renal morphology is unlikely to respond to Estrogens, so we initially chose

not to pursue this model for this indication (*5*). We later went back and tested these cells grown in three-dimensional cysts. These cysts showed good polarization. Spindle orientation was highly regulated, and spindles became misoriented upon disruption of actin (using Latrunculin) and disruption of astral microtubules (using nanomolar dose nocodazole), as expected. Spindle orientation was also disrupted upon EGTA treatment, implicating a role for cell-cell or cell-matrix junctions. Further experiments to distinguish these two types of junctions are ongoing. However, spindle orientation was not affected by treatment with Estradiol or with the potential downstream acting hepatocyte growth factor/scatter factor, as had been expected (*6, 7*). Thus, we proceded to test alternative cell lines.

We tested MCF-7 breast cancer cells, as well as an ovarian cancer cell line, but we chose not to use these because they did not polarize well. We acquired a uterine cancer cell line, Ishikawa, which expresses the Estrogen Receptor and is reported to polarize well in culture, but this also showed a great deal of variability in spindle orientation (*8*). Thus, these cell lines were deemed unsuitable for the analysis.

In the meantime, we were able to culture MCF-10A cells on glass coverslips. We were surprised to find that when grown on coverslips, these cells did orient spindles with relatively good fidelity. Moreover (see section 2d), we found that Estradiol altered spindle orientation in ERIN9 cells grown on coverslips. However, repeat experiments did not show the effect. Based on these concerns, we need to repeat the experiments, trying to remove additional variables that may affect spindle orientation. Candidates include serum proteins that vary in presence and stability. Thus, the experiments will be repeated using serum free media formulations with defined components, where contributions of individual growth factors to spindle orientation can be tested independently.

2c) Establish conditions for Estradiol treatment.

Once we were able to establish a system in which MCF-10A and ERIN9 cells regulated spindle orientation, we were able to test the effects of Estradiol treatment. We tested a dose range between 50 and 200 nM, and a time-dependence from one hour to two days. There was no dose-dependence of the effect within this dose range.

Once we established conditions, we asked a related question of whether estradiol treatment altered the composition of microtubule associated proteins or the post translational modifications of tubulin in estradiol treated versus untreated cells, but we were not able to find significant differences.

2d) Grow acini in Estradiol and do immunofluorescence.

As noted above, we performed immunofluorescence of ERIN9 cells grown on glass coverslips rather than in acini. Immunofluorescence was performed using antibodies to tubulin and staining of DNA, and spindles were imaged by high-resolution confocal microscopy. Spindle angle was calculated by solving for the right triangle created by the two spindle poles and the coverslip in the X-Z projection of the image stack. This was done for ERIN9 cells treated with and without Estradiol, as well as cells treated with Estradiol plus the anti-Estrogen 4-hydroxy-Tamoxifen.

Although spindle angle was relatively regulated in these ERIN9 cells, we periodically found cells that lacked spindle regulation. We wondered whether this could be due to the lack of tight junctions in these cells. While the cells are epithelial and likely employ the integrin-based regulation of spindle orientation, they may not be able to fully take advantage of additional levels of regulation provided by the formation of junctional complexes at cell-cell boundaries (*5, 9, 10*). To address this concern, we obtained MCF-10A cells engineered to constitutively express Crumbs3, a polarity protein that induces these cells to form tight junctions (*11*). Although we have only done a preliminary analysis of these cells (see Section 2e), they appear to regulate spindle orientation more tightly than the ERIN9 or parental MCF-10A line. If this is the case, we will stably transfect these with the gene for the ER and test the effect of Estradiol on these cells as well.

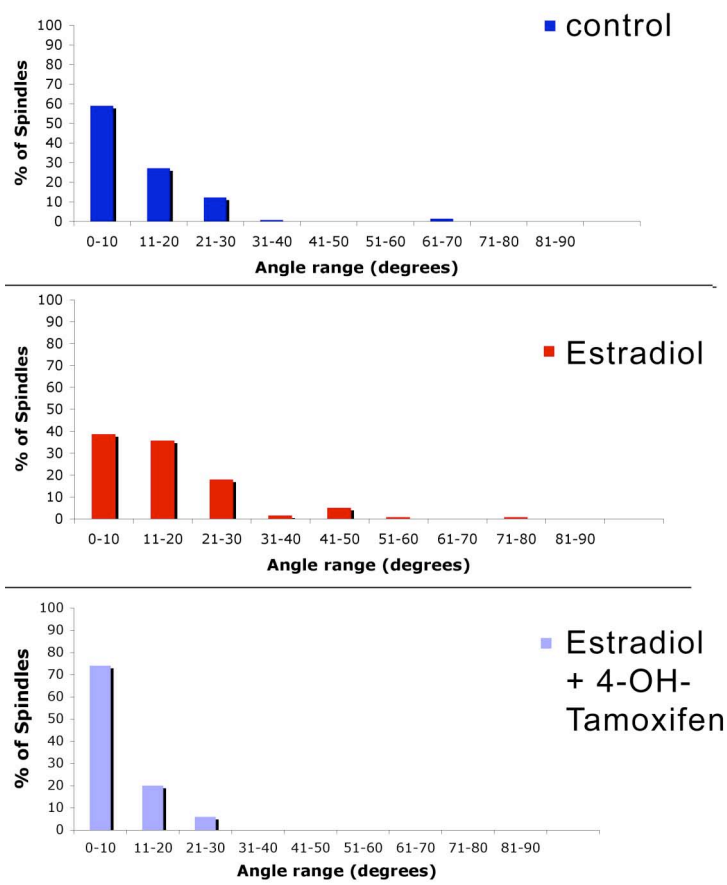

Figure 5. Estradiol treatment increases spindle angle, and the effect is inhibitable by 4-hydroxy-Tamoxifen. ERIN9 cells were treated with vehicle control (blue graph), 50 nM Estradiol (red graph) or 50 nM Estradiol plus 1 µM 4-OH-Tamoxifen (purple graph) for 24 hours. Cells were then fixed and immunofluorescence for tubulin and staining for DNA was performed. Spindle angles were analyzed by high-resolution confocal microscopy of image stacks. The angle distributions are shown for each condition, with ~150 cells analyzed for the first two graphs and 50 cells for the third. These show that spindle angle is regulated in these cells, and that the spindle angle increases in the presence of Estradiol.

2e) Image analysis from controls and Estradiol treated acini.

As noted above, we analyzed the effect of Estradiol treatment on ERIN9 cells to determine if it altered spindle orientation. We found a modest but highly reproducible effect of Estradiol treatment on spindle angle that was inhibitable by 4-hydroxy-Tamoxifen. Figure 5 shows the angle distributions, and Figure 6 shows the mean angle for each condition.

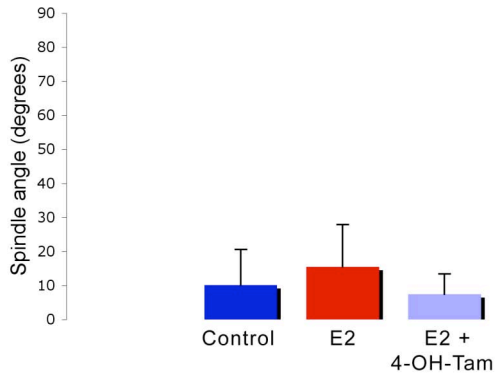

Figure 6. Summary of experiments in ERIN9 cells shows that mean spindle angle increases with Estradiol treatment. ERIN9 cells were grown on coverslips and spindle angles were measured by analysis of spindle pole position from spinning disc confocal microscopy Z-stacks in the absence (blue bar) or presence (red bar) of Estradiol, and in the presence of Estradiol plus 4-hydroxy-Tamoxifen (purple bar). Mean angle for control spindles was 10°±10° (n = 148), for Estradiol treated spindles was 16°±13° (n = 140), and for estradiol plus 4-OH-Tamoxifen was 7°±6° (n = 50). This suggests that the increase in angle was in fact due to Estrogen Receptor signaling.

Crumbs3 is a polarity protein required for formation of tight junctions in MCF-10A cells (*11*). Additionally, reduced expression of Crumbs3 in fly embryos caused spindles to become mis-oriented (*12*). We acquired an MCF-10A cell line constitutively expressing Crumbs3 for spindle angle analysis (*11*). So far, a preliminary experiment suggests that these cells regulate spindle orientation more tightly than the parental MCF-10A cell line, with a mean angle of 6° ± 5° (n = 47 spindles) (Figure 7). If further experiments confirm this result, we will stably transfect the cells with the gene for the ERα, and test the effect of Estradiol treatment as done for ERIN9 cells.

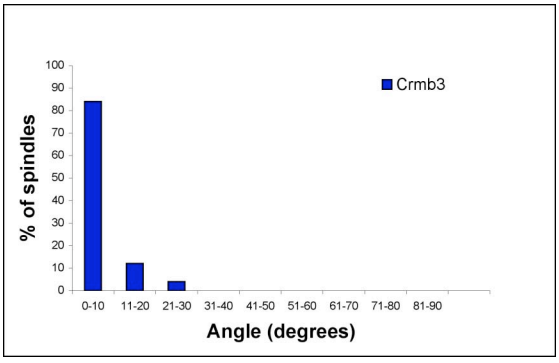

Figure 7. MCF-10A-Crumbs3 cells regulate spindle angle more tightly than the parental MCF-10A cell line. Confocal image stacks from cells plated on glass coverslips were analyzed and spindle angle calculated. Mean spindle angle was lower, at 6° ± 5° (n = 47 spindles). Also of note, the percentage of spindles with angles less than 10° was 84%, compared to the parental or ERIN9 cell line, where this typically was between 60 and 70%. This result suggests that the presence of tight junctions enhances the regulation of spindle orientation.

2f) Comparison and statistical analysis of spindle angles.

While the difference in spindle orientation caused by Estradiol is subtle, and initially seemed highly reproducible, later experiments showed variability in spindle angle. We hope to determine the source of this variability so it can be eliminated and effects of Estradiol on spindles isolated. We also hope these results will allow us to apply for larger grant funding so that we can more carefully explore the mechanisms of spindle regulation by Estradiol.

Key Research Accomplishments

- Found spindle orientation is regulated in mouse mammary tissues.
- Determined MCF-10A acini cannot be used for this study.
- Established alternate system using MCF-10A monolayers.
- Found Estradiol effect spindle orientation, inhibitable by 4-OH-Tamoxifen, but need to isolate source of variability of this effect.
- Determined spindle orientation in MDCK cysts, altered by disrupting actin, astral microtubules, and cell-cell or cell-matrix junctions.
- worked out hepatocyte growth factor conditions and found this did not affect spindle orientation in MDCK cysts.

Reportable outcomes

- Poster presentation, DoD BCRP Era of Hope meeting, June 25-28, 2008, Baltimore, MD

Conclusions

We have learned that spindle orientation is controlled in breast epithelial cells in vivo, and in breast cells grown in culture. In the mouse mammary gland, spindle orientation conforms to the ductal architecture, with spindles parallel to the duct lumen. The implications are that loss of this orientation could alter breast tissue morphology in detrimental ways, leading to pre-malignancy or cancer development. To further address the role of Estradiol in this regulation, and to determine spindle orientation in tumors, we need to do two things. First, we need to obtain human samples with adequate proliferation to measure spindle angles. This is underway. Second, we need to expand our use of mouse tissues to include tumor samples. This will tell us whether spindles are mis-oriented in tumors, and the mis-orientation can be correlated with ER status. This is also underway.

In cultured cells, spindles are orientated parallel to the coverslip, consistent with planar spindle orientation that could be controlled by several mechanisms. Estradiol appears to modestly reduce this control of spindle orientation, causing larger spindle angles; this effect is inhibitable by 4-hydroxy-Tamoxifen. We will continue our work to increase the signal and reduce the noise of this effect. We will conduct further studies to determine its mechanism, and its consequences. These studies will greatly inform our understanding of Estrogen action. They have the potential to lead to more informed use of anti-Estrogen therapies as well as therapies that improve cell polarization and mitotic spindle orientation as a means to reduce breast cancer risk and progression.

Significance ("So What…"):

Our preliminary findings that mitotic spindle orientation is regulated by Estrogen will be useful for breast cancer researchers in several ways. First, it connects Estrogen Receptor (ER) signaling to a previously unconnected pathway, a finding that will inspire studies into its mechanisms. These studies may help explain how ER signaling contributes to the etiology of breast cancer. Second, it links ER signaling to basic determinants of breast tissue morphology, suggesting that spindle orientation defects could induce pre-malignant changes. As such, these changes could be used in diagnostic testing for breast cancer risk, and they could serve as targets for breast cancer risk reduction. Finally, this work will inspire searches for interventions that can correct spindle mis-orientation caused by Estradiol exposure. These may be useful tools both for understanding breast cancer progression and for treating breast cancer.

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Appendices

There are no appendices.